Doctoral thesis

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Probiotics in Oral Health:

Interactions between Probiotics and Oral Biofilms

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals.

I. Jiang Q, Stamatova I, Kari K, Meurman JH. Inhibitory activity *in vitro* of probiotic lactobacilli against oral *Candida* under different fermentation conditions. Beneficial Microbes. 2015;6:361-368.

II. Jiang Q, Stamatova I, Kainulainen V, Korpela R, Meurman JH. Interactions between *Lactobacillus rhamnosus* GG and oral micro-organisms in an in vitro biofilm model. BMC Microbiology. 2016;16:149.

III. Jiang Q, Kainulainen V, Stamatova I, Korpela R, Meurman JH. *Lactobacillus rhamnosus* GG in experimental oral biofilms exposed to different carbohydrate sources. Caries Research. 2018;52:220-229.

IV. Jiang Q, Kainulainen V, Stamatova I, Janket S, Meurman JH, Korpela R. Mouthwash effects on LGG-integrated experimental oral biofilms. (*accepted for publication by Dentistry Journal, 2020*)

ABBREVIATIONS

4SP	Biofilm group with *Streptococcus sanguinis*, *Aggregatibacter actinomycetemcomitans*, *Candida albicans*, and *Fusobacterium nucleatum*
4SP+LGG	Biofilm group with *S. sanguinis*, *A. actinomycetemcomitans*, *C. albicans*, *F. nucleatum*, and LGG
5SP	Biofilm group with *S. sanguinis*, *A. actinomycetemcomitans*, *C. albicans*, *F. nucleatum*, and *S. mutans*
5SP+LGG	Biofilm group with *S. sanguinis*, *A. actinomycetemcomitans*, *C. albicans*, *F. nucleatum*, *S. mutans*, and LGG
Aa	Biofilm group with *A. actinomycetemcomitans*
ATCC	American type culture collection
BHI	Brain Heart Infusion
BOP	Bleeding on probing
Ca	Biofilm group with *C. albicans*
CFU	Colony-forming unit
CLSM	Confocal laser scanning microscopy
EIR	Effective inhibition ratio
FISH	Fluorescence *in situ* hybridization
Fn	Biofilm group with *F. nucleatum*
GI	Gingival index
HA	Hydroxyapatite
LGG	*Lactobacillus rhamnosus* GG or LGG®
MMP-3	Matrix metalloproteinase-3
MPO	Myeloperoxidase
MRS	De Man, Rogosa, and Sharpe medium
MS	Mutans streptococci
O/N	Overnight
OD	Optical density
PD	Pocket/probing depth
PI	Plaque index
PS	Physiological saline
Sm	Biofilm group with *S. mutans*
SRP	Scaling and root planing
Ss	Biofilm group with *S. sanguinis*

ABSTRACT

In the following series of *in vitro* studies, we aimed to investigate the interactions between probiotic lactobacilli and opportunistic oral pathogen species under various conditions. Our study hypothesis was that probiotics integrate into oral biofilms, interact with species in the biofilms, and thus alter their pathogenic potential, which further reduces the risk for some common infectious oral diseases. Investigations on probiotic properties in the presence of oral pathogens *in vitro* could facilitate and expand our understanding of inherent modes of probiotic activity.

First, we evaluated the inhibitory activity of six commercial probiotic lactobacilli against *Candida albicans* and non-*albicans Candida* with an agar-overlay method. The inhibitory activity of probiotic lactobacilli against *C. albicans* was strain-dependent and varied according to pH and carbohydrate source. *Lactobacillus rhamnosus* GG (LGG) and *C. albicans* were the species we selected for the sub-studies that followed, because LGG showed the strongest inhibitory activity, and *C. albicans* was the most susceptible yeast.

Second, we assessed the potential risk for lactobacilli on dental hard tissues by investigating the pH of the spent culture medium of lactobacilli in planktonic cultures and in single-species biofilms, as well as when co-cultured with other microbes. In five-species biofilms (5SP) in the presence of LGG, LGG was able to grow well with lactose or sucrose as its only carbohydrate source. LGG did not significantly reduce the pH when cultivated with the other five species of oral pathogens in biofilms, nor did the carbohydrate source affect the pH values.

Third, we investigated the growth of (opportunistic) oral pathogens in the presence or absence of LGG in dual- and multi-species biofilms under five carbohydrate conditions. Probiotic LGG promoted the growth of *Streptococcus mutans* and of *S. sanguinis* compared to their growth without LGG; LGG suppressed, however, the growth of *C. albicans*; and *C. albicans* and *S. mutans* significantly promoted the growth of LGG. LGG showed no impact on the growth of the pathogens in group

5SP+LGG (including all of the pathogens tested), but LGG was able to reduce the growth ratio of *S. sanguinis* in four-species biofilms+LGG (including all of the tested pathogens except *S. mutans*).

Further evaluated was also the susceptibility of biofilm species to commercial mouthwashes. LGG neither enhanced nor weakened the mouthwash antimicrobial effects on the pathogens. Their recovery after mouthwash rinsing was not influenced by the residual LGG.

In conclusion, LGG could inhibit the growth of *C. albicans*; however, *C. albicans* promoted the growth of LGG. Differing from the traditional understanding, LGG growing with multi-species biofilms could survive and grow well under conditions in which lactose or sucrose was the only carbohydrate source. Probiotic LGG and pathogenic streptococci could benefit each other when co-cultured in dual-species biofilms, but this phenomenon did not occur in multi-species biofilms. The probiotics showed no influence on the antimicrobial effect of mouthwashes.

1 INTRODUCTION

The term “probiotic”, first mentioned in 1965, has developed to mean “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [Hill *et al.*, 2014]. Probiotics are often added to ordinary fermented food, such as yoghurt, bread, or kimchi. They can also be found in drugstores as food supplements in various forms such as tablets, drops, and capsules.

Growing evidence has shown that consuming probiotics may alleviate some of the symptoms of oral conditions caused by imbalances in oral microbial homeostasis. For instance, intake of probiotics has reduced the count of salivary mutans streptococci (MS), of periodontal pathogens, and of *Candida* yeasts, and has reduced the plaque index, gingival bleeding on probing, periodontal pocket depth, and gingival inflammation [Ince *et al.*, 2015; Ghasemi *et al.*, 2017; Miyazima *et al.*, 2017].

The underlying mechanisms of the benefits are still unclear, however. Probiotics have shown inhibitory effects on (opportunistic) pathogens, which is also one of the most important criteria for screening new promising probiotics. Many *in vitro* studies have repeatedly and conclusively shown that probiotic lactobacilli inhibit the growth of MS and periodontal pathogens, but only recent studies have investigated the growth inhibition by probiotics against *Candida* in laboratory conditions, especially in biofilm models.

Multi-species biofilms are natural living formats of microorganisms, showing far stronger resistance to inhibitory substances than shown by planktonic cells [Orazi and O'Toole, 2019]. Few studies have, however, tested the growth of oral (opportunistic) pathogens in the presence of probiotics in dual-species biofilms or in multi-species biofilms.

In addition, the safety of probiotics is of major concern. Probiotic lactobacilli generate acids, a process that may pose potentially harmful effects on dental hard tissues. Some studies found that probiotic lactobacilli have led to mineral loss in

tooth enamel [Schwendicke *et al.*, 2014], but some have disputed this finding [Pham *et al.*, 2011].

Most probiotic-pathogen studies focus more on the growth of pathogens, because the amount of probiotics administered is adjustable based on need. The potential growth of probiotics is essential to determine, however, prior to deciding upon their optimal amounts in preparations and the format of their delivery.

In daily life, individuals use more than one method to prevent biofilm-related oral diseases. Conflicts may appear in the combination of chemical and biological methods. Few studies have investigated, for instance, the influence of mouthwash use on probiotic efficacy, and the effect of probiotics on the efficacy of mouthwashes.

This thesis therefore aimed to investigate how probiotic lactobacilli and oral (opportunistic) pathogens grow in experimental biofilms and to assess their mutual interactions under differing conditions. The hypothesis was that probiotics integrate into oral biofilms, interact with species in the biofilms, and alter its pathogenic potential, all of which further reduce the growth of biofilms and thus reduce the burden of common infectious oral diseases.

2 REVIEW OF THE LITERATURE

2.1 Oral microbes and microbial biofilms

2.1.1 Oral microbes

The expanded Human Oral Microbiome Database has claimed that 771 microbial species, including cultivated and uncultivated phylotypes, are detectable in the human aerodigestive tract (www.ehomd.org) [Chen *et al.*, 2010]. The diversity of microbes inhabiting the oral cavity is mainly affected by age, food intake, individual genetics, and immune defenses, and also affected by intake of antimicrobial agents. Furthermore, because of the various niches of the oral cavity, the composition of microbes differs between those on the surface of the teeth, in the gingival crevices, on the tongue, cheeks, lips, and the hard and soft palates. Via the inflamed periodontal pockets, these microbes can enter the bloodstream and reach distant parts of the body.

2.1.2 Biofilms

In nature, microbes live as planktonic cells in liquid and air and as biofilms on surfaces. Biofilms were first described in a report by the Dutch merchant Antonie van Leeuwenhoek: “the number of these animalcules in the scurf of a man’s teeth are so many that I believe they exceed the number of men in a kingdom.” In the last two decades, research on biofilms became a serious scientific endeavor in microbiology. Now we know that a biofilm is a structured consortium of microbes embedded in a self-produced polymer matrix mainly consisting of polysaccharides, proteins, nucleic acids, and lipids [Flemming and Wingender, 2010; Hoiby *et al.*, 2010].

Without any oral hygiene practice, the biofilm starts forming in the oral cavity within four hours after each meal [Palmer *et al.*, 2003]. The first colonizers, usually streptococci, attach to the saliva pellicle on the surface of the tooth or mucosal epithelium or on both, and then come the next colonizers, and gradually they form

the organized multispecies community that interacts within the microbial members and with the environment [Marsh, 2005; Verma *et al.*, 2018].

2.2 Biofilm-associated oral diseases

When the balance of the microbial ecology in the oral cavity is perturbed for a certain period, biofilm-related poly-microbial diseases may occur, for example dental caries, periodontal diseases, and yeast infections.

2.2.1 Dental caries

The World Health Organization (WHO) has claimed that dental caries is the most common non-communicable disease worldwide and is the leading pathology in the permanent teeth (2.3 billion people affected) [WHO., 2017]. Dental caries is a multifactorial outcome of the complex interactions among the caries-associated microbes, a long-term frequent supply of fermentable carbohydrates, plus susceptible tooth surfaces without proper oral hygiene. Recent molecular-approach-based studies have clearly shown that dental caries is a poly-microbial-associated disease [Simon-Soro and Mira, 2015]. When the balance of demineralization and remineralization shifts towards a net mineral loss, acid-producing microbes, for example, MS, non-MS, and *Actinomyces*, greatly contribute to the development of caries. *S. mutans*, especially, is considered to play a major etiological role in the caries progression, due to its ability to adhere to the tooth surface, by producing sticky extracellular polysaccharides from sucrose, and by fermenting sucrose and other sugars to acids, which cause mineral loss from the tooth enamel [Hamada and Slade, 1980].

2.2.2 Periodontal diseases

Periodontal diseases, with a high prevalence affecting up to 90% of the world's population, are clinically manifested as inflammation of the soft tissues around the teeth [Pihlstrom *et al.*, 2005]. Clinical observations have indicated that periodontal diseases are associated with accumulated dental biofilms adjacent to the gingival

crevices and in periodontal pockets. Furthermore, individuals with gingivitis and periodontitis may be at higher risks for the chronic inflammatory and systemic diseases, for example cardiovascular diseases, stroke, Alzheimer's disease, and diabetes mellitus [Söder *et al.*, 2015; Beukers *et al.*, 2017; Pritchard *et al.*, 2017; Graziani *et al.*, 2018]. Generally, the most routinely isolated bacteria are *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum*. A great number of previously unknown pathogens are being identified with new molecular and sequencing techniques [Hiranmayi *et al.*, 2017].

2.2.3 Yeast infections

An oral yeast infection known as thrush or candidiasis, is common and is caused by the genus *Candida*. Many of the *Candida* strains live as commensal strains in the host, but in immunocompromised individuals, their virulence and pathogenic properties may be enhanced, leading to clinical manifestations like chronic mucosal infections, and even to life-threatening systemic diseases. These diseases are most frequently induced by *Candida albicans*, but also by non-*albicans Candida* strains, for example *C. glabrata*, *C. krusei*, and *C. tropicalis* [Hani *et al.*, 2015].

2.3 Oral hygiene

To prevent biofilm-associated oral diseases by reduction or control of the accumulated dental biofilms, the basic practice for individuals is appropriate oral self-care [Kumar *et al.*, 2016; Sicca *et al.*, 2016; Lertpimonchai *et al.*, 2017]. Home-based oral hygiene can include mechanical cleaning such as by toothbrushing, and also including chemical cleaning, such as with antiseptic mouthwashes.

2.3.1 Mechanical cleaning

Mechanical cleaning normally includes cleaning of the teeth and interdental spaces, oral mucosa, and tongue with a brush, dental floss, and a moist cotton swab. Mechanical cleaning is considered to be the most reliable procedure for plaque

removal, which is essential for the prevention of oral diseases. However, dental biofilms are nearly impossible to remove completely, such as those in gingival margins, fissures, buccal pits, and in the posterior interproximal areas, where oral diseases mostly develop [Sheiham and Sabbah, 2010; Jongsma *et al.*, 2015].

2.3.2 Chemical cleaning

Chemical cleaning refers to rinsing with mouthwashes to clean the teeth and oral mucosa relying on the antimicrobial and anti-biofilm-forming properties of these products. Mouthwash rinse usually serves as a support to mechanical cleaning. It can also be the only oral care for patients who are unable to brush their teeth, and who have, for example, recently undergone surgery, or for individuals with motor or cognitive impairments [Tartaglia *et al.*, 2017]. Of the antiseptic components, chlorhexidine is the gold standard, which is recommended only for the short-term use because of its potential adverse effects, like tooth discoloration [Zanatta *et al.*, 2010].

2.4 Probiotics

2.4.1 History

In 1905, Stamen Grigorov, a Bulgarian physician, identified a species of *Lactobacillus* in the starter culture of a fermented Bulgarian dairy product. Based on this finding, in the following year the later Nobel laureate Élie Metchnikoff published a book *Prolongation of life: Optimistic studies*, which postulated that the cause underlying the longer life of Bulgarian peasants was the yogurt that they consumed, containing health-beneficial bacteria [Ozen and Dinleyici, 2015]. Henry Tissier, in 1900, was the first scientist to conceive the idea that friendly bacteria could be useful in treating intestinal diseases [Farré-Maduell and Casals-Pascual, 2019]. Today, those microorganisms are indeed called “probiotics”.

2.4.2 Definition

The meaning of “probiotic” is “fit for life, lively.” The term was defined as “growth promoting factors produced by microorganisms” according to Lilly and Stillwell [1965]. After many decades of evolution, probiotics now are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [Hill *et al.*, 2014]. The hosts can be humans, animals [Yang *et al.*, 2017], or plants [Kim and Anderson, 2018]. Probiotic strains that have demonstrated human-health benefits mainly belong to the genera of *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Bifidobacterium*, *Bacillus*, *Escherichia coli*, and *Saccharomyces* [Fijan, 2014].

2.4.3 Probiotics and general health

The human body harbors more microbial cells than human cells [Sender *et al.*, 2016]. Microbial cells reside in such areas as the gastrointestinal tract, skin, hair, external auditory canal, nostrils, vagina, and penis. [Blum, 2017]. A balanced microbial ecosystem is essential to physiology, immunity, and mental health. Once the balance shifts towards an unhealthier situation, dysbiosis symptoms, or diseases, or both may appear. Antimicrobial therapy has prolonged the human life span, beginning with the detection of penicillin. However, nowadays, one of the greatest threats to global life is antibiotic resistance. Alternative prevention methods and treatments are thus urgently needed. Probiotics have drawn the attention of researchers and medical professionals because of usually showing no adverse effects.

Knowledge of probiotic effects on microbial diseases is accumulating. What has been clinically demonstrated is that probiotic intervention proves effective for the prevention and treatment of certain gut diseases such as infectious diarrhea, antibiotic-associated diarrhea, inflammatory bowel diseases, irritable bowel syndrome, *Helicobacter pylori* infection, and lactose intolerance [Sanchez *et al.*, 2017]. Probiotics have shown promise in improving the vaginal environment by maintaining its normal lactobacilli count [Vladareanu *et al.*, 2018]. They have also

helped to prevent allergies [Fiocchi *et al.*, 2015], to improve acne vulgaris and atopic dermatitis [Jung *et al.*, 2013; Fabbrocini *et al.*, 2016; Prakoeswa *et al.*, 2017; Zhao *et al.*, 2018], to ease postpartum depression and anxiety [Slykerman *et al.*, 2017], and to offer other benefits. These benefits are believed to be strain specific. Zmora et al. [2018] suggest that probiotic products should be tailored to each individual's specific health needs, rather than prescribing one product to all.

Knowledge of the exact mechanisms of probiotic action is, however, limited. Locally, it may include a competition with pathogens for adhesion sites and nutrients, or include inhibition of the pathogens' growth by secretion of bacteriocins and similar products, therefore modulating the composition and activity of the indigenous microorganisms. Furthermore, probiotics' local beneficial effects may enhance epithelial barrier function, modulate the immune system and its systemic metabolic responses by signaling via the central nervous system, and also may regulate human general health and physiology [Sommer and Backhed, 2013; Lebeer *et al.*, 2018].

2.4.4 Probiotics and oral health

Probiotics and dental caries

A reduced count of salivary MS is regarded as a major indicator of reduced risk for caries in clinical trials (**Table 1**). Short- and long-term intakes of probiotics have shown the potential to prevent caries and to reduce caries development. The positive effects of probiotics could be as efficient as chewing xylitol-containing gum [Ghasemi *et al.*, 2017]. MS-inhibiting results have also appeared from *in vitro* studies [Conrads *et al.*, 2019; Nunpan *et al.*, 2019].

The mechanism of the beneficial effects may be a shifting of the microbial ecology in the oral cavity towards a more beneficial state of equilibrium producing fewer organic acids. One mechanism of action causing inhibition of growth, of biofilm formation, or of the virulence properties of MS, –or any combination of these–

involves bioactive substances released by probiotics [Rossoni *et al.*, 2018b; Wasfi *et al.*, 2018].

Probiotic interventions have, however, also failed to result in any significant change in plaque and salivary MS counts [Cildir *et al.*, 2012; Keller *et al.*, 2012; Marttinen *et al.*, 2012; Villavicencio *et al.*, 2018]. One suggestion is that probiotic intervention to prevent caries may prove more effective in children at high risk for caries [Rodriguez *et al.*, 2016; Villavicencio *et al.*, 2018]. Moreover, who differ in their eating habits, ethnicities, ages, or locations may harbor greatly differing oral microorganisms, and may respond differently to any specific probiotic administration [Percival *et al.*, 1991; Sampaio-Maia and Monteiro-Silva, 2014; Gupta *et al.*, 2017; Lira-Junior *et al.*, 2018]. This indicates that probiotic administration should indeed be tailored to each individual.

Table 1. Clinical effects of commercial probiotic products/strains on dental caries and related conditions.

Strain(s)	**Delivery format**	**Test duration/ Tested population**	**Outcome**	**Reference**
Lactobacillus rhamnosus SP1	Milk	10 months/2-3 yrs with high risk for caries	Reduced caries development	[Rodriguez *et al.*, 2016]
L. rhamnosus CGMCC 1.3724, and *Bifidobacterium longum* ATCC BAA-999	Milk	9 months/3-4 yrs	No significant reduction in *Streptococcus mutans* counts, no caries-preventive effect	[Villavicencio *et al.*, 2018]
LGG	Milk	7 months/1-6 yrs	Reduced dental caries and lower MS counts; effects particularly clear in children aged 3 or 4 years	[Nase *et al.*, 2001]

Strain(s)	Delivery format	Test duration/ Tested population	Outcome	Reference
L. rhamnosus LB21	Milk	15 months/58-84 yrs	Root caries index reversal occurred, and electric resistance measurement values increased, indicating remineralization. Both effects enhanced with fluoride. No significant alteration in microbial counts	[Petersson *et al.*, 2011]
L. paracasei SD1	Milk powder	4 weeks/18-25 yrs	Reduction in salivary MS counts	[Teanpaisan and Piwat, 2014]
L. rhamnosus LB21	Milk with fluoride	21 months/1-5 yrs	Milk-containing probiotics reduced caries development	[Stecksen-Blicks *et al.*, 2009]
L. acidophilus ATCC 4356 and *B. bifidum* ATCC 2951	Yogurt	3 weeks/19-27 yrs female	Reduction in salivary MS counts, as effective as chewing gum with xylitol	[Ghasemi *et al.*, 2017]
L. reuteri DSM 17938 and *L. reuteri* ATCC PTA 5289	Drop	25 days/4-12 yrs operated children with cleft lip or palate	No significant changes in salivary MS and lactobacilli counts	[Cildir *et al.*, 2012]
L. reuteri ATCC 55730	Freeze-dried powder in oil drops (mixed refined coconut oil and peanut oil, 3:1)	1 + 12 months/ expectant mothers during final month of gestation and newborn babies from families with allergic-disease history	Reduced caries prevalence at age 9 years, but no significant changes in MS and lactobacilli counts in saliva and plaque, or in secretory IgA	[Stensson *et al.*, 2014]
B. lactis BB-12 and *L. acidophilus* La-5	Ice-cream	10 days/12-14 yrs with no detectable clinical caries	Reduction in salivary MS counts	[Singh *et al.*, 2011]
L. paracasei F19	Cereal	9 months/ 4-month-old full-term infants	No effects on prevention of caries at ages 3, 6, 9 years, no effect on salivary MS or lactobacilli at age 9	[Hasslof *et al.*, 2013]

Strain(s)	Delivery format	Test duration/ Tested population	Outcome	Reference
LGG	Tablet	2 weeks/25.3±2.4 yrs	No significant changes in plaque MS counts and no significant alteration of acidogenicity of supra-gingival plaque. No significant change in plaque lactobacilli	[Marttinen *et al.*, 2012]
L. reuteri SD2112 and *L. reuteri* PTA 5289	Tablet	2 weeks/25.3±2.4 yrs	No significant changes in plaque MS counts and no significant alteration of acidogenicity of supra-gingival plaque. Significant increase in plaque lactobacilli	[Marttinen *et al.*, 2012]
B. animalis subsp. *lactis* BB-12	Tablet	until 2 yrs old/ 1-2-month-old babies	No effects on caries occurrence at age 4 in a low-caries population	[Taipale *et al.*, 2013]
S. uberis KJ2, *S. oralis* KJ3, and *S. rattus* JH145	Chewing tablet	1 year/2-3 yrs	Reduced risk for early childhood caries development	[Hedayati-Hajikand *et al.*, 2015]
L. reuteri DSM 17938 and *L. reuteri* ATCC PTA 5289	Lozenge	6 weeks/19-35 yrs with moderate or high counts of salivary MS	No effect on regrowth of MS after full-mouth disinfection with chlorhexidine	[Keller *et al.*, 2012]
L. paracasei ADP-1	Tooth-paste	Brushing for 30 s and swishing for 30 s/10-12 yrs with no caries	Reduced MS mono-species biofilms. Effect short, when exposed to sucrose	[Srinivasan *et al.*, 2017]

MS: mutans streptococci; yrs: years; s: seconds.

Probiotics and periodontal diseases

Tlaskalova-Hogenova *et al.* [2004] have indicated that probiotics can prevent or treat chronic diseases because the regulation of microbial composition affects the development of mucosal and systemic immunity. Beneficial effects of probiotics on periodontal diseases have been confirmed in a number of clinical studies (**Table 2**), but the mechanisms remain poorly understood.

Until now, the most extensively studied probiotic genus for periodontal health has been *Lactobacillus*, specifically the species *L. reuteri.* Almost all the published

clinical studies using *L. reuteri* (mainly Prodentis® lozenges, BioGaia, Lund, Sweden) showed positive effects on periodontal disease parameters. For example, a reduced gingival index was evident [Ince *et al.*, 2015; Tekce *et al.*, 2015], as well as reduced pocket depth [Teughels *et al.*, 2013; Vicario *et al.*, 2013; Ince *et al.*, 2015; Tekce *et al.*, 2015], reduced counts of inflammatory pathogens [Iniesta *et al.*, 2012; Teughels *et al.*, 2013], and improved attachment gain [Teughels *et al.*, 2013]. The positive effects may be due to reuterin, a bacteriocin produced *by L. reuteri*. Reuterin has the ability to induce oxidative stress in cells, thereby showing bacteriostatic and bactericidal activities against a wide range of pathogenic microorganisms [Schaefer *et al.*, 2010].

Promising results have also emerged from probiotic studies with *L. plantarum* L-137 [Iwasaki *et al.*, 2016], *L. brevis* CD2 [Shah *et al.*, 2017], and *L. salivarius* WB21 [Shimauchi *et al.*, 2008]. Heat-killed *L. plantarum* L-137 has proven to be a potent inducer of interleukin-12 [Murosaki *et al.*, 1998], which leads to a T-helper-1-type immune response through induction of interferon-β and interferon-γ [Murosaki *et al.*, 1999; Arimori *et al.*, 2012]. Riccia *et al.* [2007] suggested that the beneficial effect of *L. brevis* may result from arginine deiminase, which prevents nitric oxide generation. The administration, in sachets, of *L. rhamnosus* SP1, the effect of which was believed to be similar to the effect of azithromycin, did not, however, demonstrate better effects than did mechanical scaling and root planing alone [Morales *et al.*, 2016; 2018]. The reasons are unclear, but delivery format, dosage, and frequency of administration may also influence the intervention outcomes.

During the past six years, researchers' interest has increased in studying another commonly used probiotic genera, namely *Bifidobacterium*. *Bifidobacterium* alone [Kuru *et al.*, 2017; Invernici *et al.*, 2018] and mixed with lactobacilli [Toiviainen *et al.*, 2015; Montero *et al.*, 2017; Yousuf *et al.*, 2017; Alanzi *et al.*, 2018; Becirovic *et al.*, 2018] underwent various investigation of their ability to reduce the risk for periodontal disease. All tests resulted in positive effects. In one *in vitro* study, Jasberg *et al.* [2016] demonstrated that the *Bifidobacterium animalis* subsp. *lactis* BB-12 showed an inhibitory effect on the growth of *Porphyromonas gingivalis*.

However, administration of a combination of three species of *Streptococcus*, as investigated by Laleman *et al.* [2015] did not achieve the expected benefits concerning periodontal disease, based on clinical and microbiological parameters, although some streptococci seemed to reduce the inflammatory response induced by pathogenic microorganisms *in vitro* and *in vivo* [Zhang and Rudney, 2011; Kaci *et al.*, 2014]. Alkaya *et al.* [2017] and Tsubura *et al.* [2009] have also reported inconsistent results from *Bacillus* intervention. In a rat model with ligature-induced periodontitis, probiotic *Bacillus* reduced attachment loss, alveolar bone loss, and tissue breakdown [Messora *et al.*, 2013; Foureaux Rde *et al.*, 2014]. Such inconsistent results may be the result of the probiotic concentration, delivery format, or parameter measurements.

Table 2. Clinical effects of commercial probiotic products/strains on periodontal diseases and related conditions.

Strain(s)	**Delivery format**	**Test duration/ Tested population**	**Outcome**	**Reference**
L. casei Shirota	Milk	8 weeks/mean age 24.4±1.9	Decreased elastase activity and MMP-3 amount. Increased MPO activity	[Staab *et al.*, 2009]
Bifidobacterium animalis subsp. *lactis* BB-12, *L. acidophilus* La-5 and LGG	Milk	3 weeks/dental students	Reduction of periodontal pathogens	[Becirovic *et al.*, 2018]
B. animalis subsp. lactis DN-173010	Yogurt	28 days/16-26 yrs	Reduced plaque accumulation and gingival inflammation	[Kuru *et al.*, 2017]
L. reuteri ATCC 55730	Freeze-dried powder in oil drops (mixed refined coconut oil and peanut oil, 3:1)	1 + 12 months/ expectant mothers during final month of gestation and newborn babies from families with allergic-disease history	Reduced gingival bleeding index at age 9, no significant change in PI	[Stensson *et al.*, 2014]

Strain(s)	**Delivery format**	**Test duration/ Tested population**	**Outcome**	**Reference**
L. reuteri ATCC 55730 and *L. reuteri* ATCC PTA 5289 (Prodentis®)	Tablet	30 days/≥18 yrs	Decreased PI, BOP, and PD	[Vicario *et al.*, 2013]
L. reuteri DSM17938 and *L. reuteri* ATCC PTA 5289 (Prodentis®)	Tablet	8 weeks/20-24 yrs	Reduced counts of periodontal pathogens	[Iniesta *et al.*, 2012]
L. salivarius WB21	Tablet	8 weeks/32-61 yrs	Reduced PI, PD, and salivary lactoferrin	[Shimauchi *et al.*, 2008]
L. plantarum CECT 7481, *L. brevis* CECT 7480, and *Pediococcus acidilactici* CECT 8683	Tablet	6 weeks/18-55 yrs	Reduction in number of sites with severe inflammation	[Montero *et al.*, 2017]
S. oralis KJ3*, S. uberis* KJ2*,* and *S. rattus* JH145	Tablet	12 weeks/37-58 yrs with SRP	No significant differences in clinical nor microbiological parameters, but decreased number of sites with plaque, and decreased counts of *Prevotella intermedia*	[Laleman *et al.*, 2015]
L. brevis CD2	Lozenge	14 days/14-35 yrs with aggressive periodontitis	Reduced GI. Effect equivalent to that of doxycycline	[Shah *et al.*, 2017]
LGG and *B. lactis* BB-12	Lozenge	4 weeks/13-15 yrs	Reduction in GI. Reduction in periodontal pathogens (saliva and plaque *A. actinomycetemcomitans*, *P. gingivalis*)	[Alanzi *et al.*, 2018]
L. reuteri	Lozenge	360 days/35-50 yrs with SRP	Decreased PI, GI, BOP, and PD. Reduction in inflammatory markers	[Ince *et al.*, 2015]
L. reuteri DSM17938 and *L. reuteri* ATCC PTA 5289 (Prodentis®)	Lozenge	360 days/mean age 43±5.01, and 41.4±8.86 with SRP	Decreased PI, GI, BOP, and PD	[Tekce *et al.*, 2015]

Strain(s)	Delivery format	Test duration/ Tested population	Outcome	Reference
L. reuteri DSM17938 and *L. reuteri* ATCC PTA 5289 (Prodentis®)	Lozenge	12 weeks/≥35 yrs with SRP	Decreased PD, improved attachment gain, and reduction in periodontal pathogens	[Teughels *et al.*, 2013]
LGG and *B. animalis* subsp. *lactis* BB-12	Lozenge	4 weeks/mean age 24.6±2.7, 24±3.0 yrs	Decreased PI and GI	[Toiviainen *et al.*, 2015]
B. animalis subsp. *lactis* HN019	Lozenge	30 days/≥30 yrs with treatment of SRP	Decreased PD, improved attachment gain, reduced counts of periodontal pathogens, and lower pro-inflammatory cytokine levels	[Invernici *et al.*, 2018]
L. rhamnosus SP1	Sachet	3 months/≥35 yrs with SRP	No significant differences in clinical and microbiological improvements	[Morales *et al.*, 2016; 2018]
L. plantarum L-137	Capsule containing heat-killed strains	12 weeks/mean age 66.2 yrs	Decreased PD	[Iwasaki *et al.*, 2016]
Bacillus subtilis, *Ba. megaterium*, and *Ba. pumilus*	Tooth-paste/ mouth rinse/ toothbrush cleaner	8 weeks/18-31 yrs	No significant differences in clinical parameters	[Alkaya *et al.*, 2017]
Ba. subtilis E-300	Mouth-wash	1 month/44-62 yrs	Reduction in GI, and in periodontal pathogens	[Tsubura *et al.*, 2009]
L. acidophilus, B. longum, B. bifidum, and B. lactis	Freeze-dried powder in water as mouth-rinse	3 weeks/12-15 yrs	Decreased gingival status and decreased plaque accumulation	[Yousuf *et al.*, 2017]
Lactic acid *Bacillus*	Freeze-dried powder in water as mouth-rinse	3 weeks/12-15 yrs	Decreases in gingival status and in plaque accumulation	[Yousuf *et al.*, 2017]

MPO: myeloperoxidase; MMP-3: matrix metalloproteinase-3; PI: plaque index; GI: gingival index; BOP: bleeding on probing; PD: pocket/probing depth; SRP: scaling and root planing; yrs: years.

Probiotics and yeast infections

Table 3 shows that Ahola *et al.* [2002] reported the first clinical study of probiotic use against *Candida*. In the past decade, a number of studies agreed with this *Candida*-inhibitory effect caused by probiotics administered in various delivery formats [Hatakka *et al.*, 2007; Dos Santos *et al.*, 2009; Mendonca *et al.*, 2012; Li *et al.*, 2014; Ishikawa *et al.*, 2015; Kraft-Bodi *et al.*, 2015; Miyazima *et al.*, 2017; Lee *et al.*, 2019] in adults and in the elderly. Several *in vitro* studies also have revealed that the growth and biofilm formation of *Candida* can be inhibited by probiotic cells and their supernatants [Kheradmand *et al.*, 2014; Matsubara *et al.*, 2016; Ribeiro *et al.*, 2017; Song and Lee, 2017; Rossoni *et al.*, 2018a; Tan *et al.*, 2018].

The suggested mechanisms are that probiotic *Lactobacillus* has the potential to reduce the hyphae formation and adhesion ability of *Candida*, deplete them in nutrients, and thus lessen their pathogenic potential [Basson, 2000; Krzysciak *et al.*, 2017; Tan *et al.*, 2017; de Barros *et al.*, 2018]. In addition, the antimicrobial effects of probiotics may possibly be modified by enhanced epithelial-barrier function through the induction of cytokine secretion and the production of antimicrobial substances [Bermudez-Brito *et al.*, 2012; Mendonca *et al.*, 2012].

However, the reductions in *Candida* counts were undetectable in 5- to 10-year-old caries-active children and also were undetectable in individuals with systemic oral lichen planus [Burton *et al.*, 2013; Keller and Kragelund, 2018]. The reasons for the discrepancy between studies need further clarification.

Table 3. Clinical effects of commercial probiotic products/strains on yeast infections and related conditions.

Strain(s)	**Delivery format**	**Test duration/ Tested population**	**Outcome**	**Reference**
L. casei Shirota and *B. breve* Yakult	Juice	30 days/≥65 yrs female	Reduction in *Candida* and increase in anti-*Candida* IgA	[Mendonca *et al.*, 2012]

Strain(s)	Delivery format	Test duration/ Tested population	Outcome	Reference
L. casei Shirota and *B. breve* Yakult	Yogurt	20 days/healthy young individuals	Reduction in *Candida* and in anti-*Candida* IgA	[Dos Santos *et al.*, 2009]
LGG, *L. rhamnosus* LC 705	Cheese	3 weeks/18-35 yrs	Reduction in yeast counts	[Ahola *et al.*, 2002]
LGG, *L. rhamnosus* LC 705, *Propionibacterium freudenreichiii* ssp *shermanii* JS	Cheese	16 weeks/70-100 yrs	Reduction in prevalence of high salivary *Candida* counts ($\geq 10^4$ CFU/mL), reduction in hyposalivation	[Hatakka *et al.*, 2007]
L. acidophilus NCFM or *L. rhamnosus* Lr-32	Cheese	8 weeks/elderly, complete-denture wearers	Reduction in *Candida*	[Miyazima *et al.*, 2017]
S. salivarius M18	Lozenge	3 months/5-10 yrs caries-active children	No significant reduction in saliva *Candida* counts	[Burton *et al.*, 2013]
B. longum, *L. bulgaricus* and *S. thermophilus*	Lozenge	4 weeks/18-75 yrs	Reduction in *Candida* during the treatment of *Candida*-associated stomatitis	[Li *et al.*, 2014]
L. reuteri DSM17938 and *L. reuteri* ATCC PTA 5289	Lozenge	12 weeks/60-102 yrs living in nursing home	Reduction in *Candida* in saliva and plaque	[Kraft-Bodi *et al.*, 2015]
L. reuteri DSM17938 and *L. reuteri* ATCC PTA 5289	Lozenge	16 weeks/with systemic oral lichen planus	No differences in *Candida* counts or in *Candida* carrier status	[Keller and Kragelund, 2018]
L. rhamnosus HS111, *L. acidophillus* HS101, and *B. bifidum*	Lyophilized powder	5 weeks/61.6±9.8 yrs, candidiasis-asymptomatic, denture wearers	Reduction in *Candida*	[Ishikawa *et al.*, 2015]
L. rhamnosus SP1	Sachet probiotic with milk	12 months/≥60 yrs, *Candida*-associated denture stomatitis in the elderly	Reduction in severity of denture stomatitis	[Lee *et al.*, 2019]

CFU: colony-forming units.

2.4.5 Safety issues

Due to the long history of safe consumption of probiotic-containing fermented foods, probiotics are generally recognized as safe. Despite this reassuring history, and for quality-control reasons, the safety of probiotic administration has been under continuous study. What must be taken into account are the health status of consumers, the characterization of mono- and multi-strains, the vehicle and manner of administration, and the dosage and frequency of exposure [Sanders *et al.*, 2010].

The genus *Lactobacillus* is crucial to the modern probiotic industry. Consumption of *Lactobacillus*-containing dairy products is high in France, for example, where the risk for *Lactobacillus* infection for over a century has been 5 cases per 50 million residents [Bernardeau *et al.*, 2006]. Although the pathogenesis of *Lactobacillus* infection is unknown, clinical studies make clear that use of probiotic lactobacilli has shown no adverse effects on immunologically vulnerable populations, ones including patients with HIV [Salminen *et al.*, 2004; Ishizaki *et al.*, 2017; Happel *et al.*, 2018] and groups of premature infants [Miloh, 2015]. In Finland, the increased consumption of probiotic food did not lead to any increase in the incidence of *Lactobacillus* bacteremia in the period 1995 to 2000 [Salminen *et al.*, 2002].

In addition, to evaluate the safety of probiotic use regarding oral health, probiotics need to meet not only all the requirements for their general health effects, but also they should not reduce oral pH in the presence of dietary sugars. A lowered pH favors a shift toward an acid-tolerant and acid-producing consortium of microorganisms, which alters the balance from remineralization toward demineralization, hence favoring caries progression [Takahashi and Nyvad, 2011].

As a lactic acid bacterium, *Lactobacillus* is naturally suspected of increasing the risk for dental caries. A few studies have agreed that probiotic lactobacilli have the potential to enhance the cariogenicity of *S. mutans* and to cause subsequent tooth mineral loss [Matsumoto *et al.*, 2005; Pham *et al.*, 2009; Schwendicke *et al.*, 2014]. However, Pham *et al.* in 2011 have indicated that probiotic lactobacilli are not responsible for any increase in cariogenic potential. In addition, they were even

able to reduce caries development plus reduce the risk for early childhood caries development [Nase *et al.*, 2001; Stecksen-Blicks *et al.*, 2009; Petersson *et al.*, 2011; Stensson *et al.*, 2014; Hedayati-Hajikand *et al.*, 2015; Rodriguez *et al.*, 2016]. The controversial results here discussed require further studies to reassure the public and professionals as to the safety of probiotic use in the oral cavity.

2.4.6 Delivery formats

In order to meet the differing needs of customers, probiotics on the commercial market are designed to be delivered by various vehicles and in various formats such as fermented milk, yogurt, cheese, ice-cream, meat products, fruit and vegetable juices, oats and cereals, and in other manners, with the help of drying techniques [Flach *et al.*, 2017].

Only a portion of these formats is suitable for probiotics targeting oral health, because only a few seconds are necessary for a bite of food or a swallowed drink to pass through the oral cavity. Probiotics need sufficient time to attach to oral surfaces and to interact with local microorganisms, in order to exert their direct beneficial effects.

Studies in **Tables 1**-**3** have shown that probiotics in milk, juice, yogurt, ice-cream, cheese, drop, (chewing) tablet, lozenge, sachet, capsule, toothpaste, and mouthwash all have provided positive effects against caries, periodontal diseases, or yeast infections. However, very few studies have examined whether factoring in the type of carrying format will measurably and clinically significantly influence the beneficial effects of probiotics on oral health.

3 AIMS OF THE STUDY

The general aim of this thesis was to explore how probiotic lactobacilli and oral pathogens grow in biofilms and to evaluate their mutual interactions under various conditions.

The study hypotheses were that probiotic lactobacilli are able to integrate into oral biofilms and affect their species composition, that probiotics interact with pathogens and thus affect the biofilms' pathogenic potential, and that the interactions between probiotics and oral pathogens change after treatment of the biofilms with commercially available mouthwashes.

The specific aims were

1. To evaluate the inhibitory activity of probiotic lactobacilli against *Candida*.
2. To evaluate the potential risk from lactobacilli regarding dental hard tissues in terms of pH alterations in the growth environment.
3. To assess the growth of oral pathogens in biofilms in the presence of *Lactobacillus rhamnosus* GG (LGG).
4. To assess the growth of LGG in broth and biofilms supplied with various carbohydrate sources in the presence of opportunistic pathogens.
5. To study mouthwash effects on probiotic LGG-integrated biofilms.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Probiotic lactobacilli

The study included six commercial probiotic lactobacilli (**Table 4**). These strains were stored as frozen stock in 10% skim milk at -80 °C. For each experiment, the strains were subcultured twice on de Man, Rogosa, and Sharpe (MRS) agar plates at 37 °C in 5% CO_2 for 24 h, and then the pure colonies were inoculated into MRS broth and cultivated for 16-17 h, and the cell numbers were counted. Before each test, the cells in broth were verified by Gram staining.

Table 4. Commercially available probiotic lactobacilli used in Studies I-IV.

Strains	Abbreviation	Source	Used in study
Lactobacillus rhamnosus GG	LGG	ATCC[1] 53103, Valio Ltd., Helsinki, Finland	I, II, III, IV
Lactobacillus bulgaricus LB 86	LB 86	Laboratory collection, LB Lactis, Bulgaria	I
Lactobacillus bulgaricus LB LACT	LB LACT	Laboratory collection, LB Lactis, Bulgaria	I
Lactobacillus brevis CD2	CD2	Inersan, CD Investments srl. Roma, Italy	I
Lactobacillus casei Shirota	Shirota	Yakult, Tokyo, Japan	I
Lactobacillus reuteri SD2112	SD2112	ATCC 55730	I

[1] ATCC: American Type Culture Collection.

4.1.2 Oral pathogens and opportunistic pathogens.

Table 5. Oral pathogens and opportunistic pathogens used in Studies I-IV.

Strains	Source	Medium	Air	Time	Study
Related to tooth decay					
Streptococcus mutans ATCC 27351	ATCC	BHI[1]	5% CO_2	24 h	II, III, IV
Streptococcus sanguinis ATCC 10556	ATCC	BHI	5% CO_2	24 h	II, III, IV
Related to periodontitis					
Aggregatibacter actinomycetemcomitans ATCC 43718	ATCC	BHI	5% CO_2	24 h	II, III, IV
Fusobacterium nucleatum ATCC 25586	ATCC	Brucella	Anaerobic[2]	48 h	II, III, IV
Related to yeast infection					
Candida albicans ATCC 10231	ATCC	Sabouraud	Air	O/N[3]	I, II, III, IV
Candida glabrata ATCC 90030	ATCC	Sabouraud	Air	O/N	I
Candida krusei ATCC 6258	ATCC	Sabouraud	Air	O/N	I

[1] BHI: brain heart infusion; [2] anaerobic: mixture of 0.2% O_2, 5% CO_2, 9.9% H_2, 84.9% N_2; [3] O/N: overnight.

4.1.3 Mouthwashes

Table 6. Commercial mouthwashes used in Study IV**.**

(To appear in the Open Access journal *Dentistry Journal*)

Trade name	Main active component	Manufacturer
Corsodyl®	0.2% (or 2 mg/mL) chlorhexidine gluconate	GlaxoSmithKline, UK
Listerine® Total Care	Essential oils: eucalyptol 0.092%, methyl salicylate 0.060%, thymol 0.064% and menthol 0.042%	Johnson & Johnson, UK
Meridol®	Amine fluoride and stannous fluoride (250 ppm F^-)	GABA, Switzerland

4.2 Study designs

The five aims of this thesis project were studied in the four original studies. Their interconnections are illustrated in **Figure 1**.

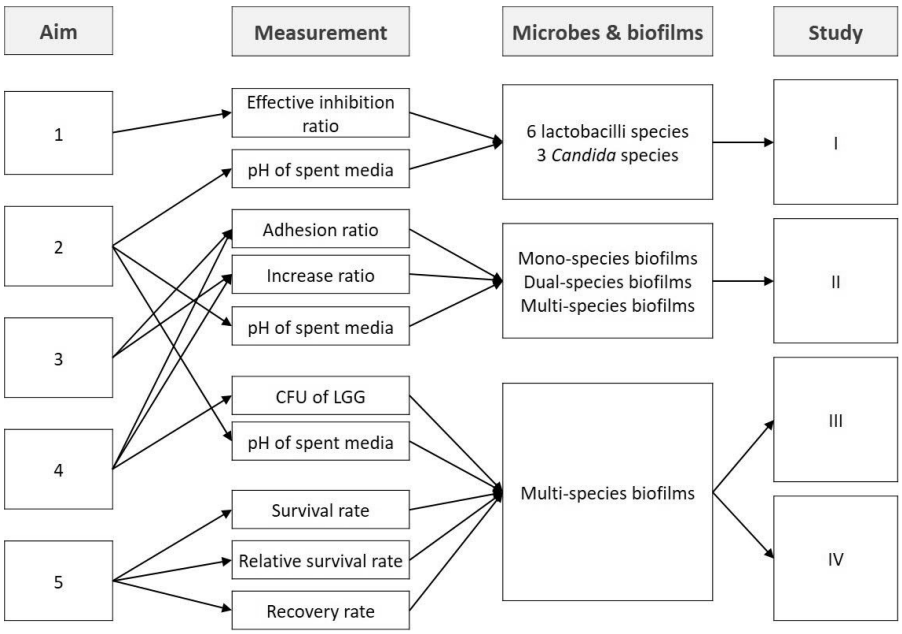

Figure 1. Overview of aims, measurements, microbes, and biofilms in Studies I-IV.

4.2.1 Inhibitory activity of probiotic lactobacilli against *Candida* (I)

We evaluated the inhibitory activity of six commercial *Lactobacillus* probiotic strains against three opportunistic oral *Candida* strains. From this study, the *Lactobacillus* species showing the strongest inhibitory activity and the *Candida* species with the highest susceptibility, LGG and *C. albicans*, were the strains selected for Studies II-IV.

4.2.2 Potential risk to dental hard tissues of lactobacilli (I-III)

The pH alterations in the growth environment served in evaluation of the potential risk to dental hard tissue.

Lactobacilli growing as planktonic cells (I)

The acid-producing abilities of lactobacilli were evaluated by measurement of the pH alteration of MRS broth containing a sole carbohydrate, namely either fructose, glucose, lactose, sorbitol, or sucrose, when inoculated with lactobacilli.

Lactobacilli growing as biofilm cells (II and III)

The acid-producing abilities of LGG were also evaluated in biofilms. First, we cultivated the biofilms of the groups Sm+LGG, Ss+LGG, Ca+LGG, 4SP+LGG, and 5SP+LGG in normal biofilm medium (with glucose as the carbohydrate source). The pH values of the spent media were measured at 16.5 h, 40.5 h, and 64.5 h. The biofilm groups without LGG served as control groups. The biofilm group composition is in **Table 7**.

Secondly, we cultivated the biofilms of group 5SP+LGG in biofilm medium with a sole carbohydrate, namely either fructose, glucose, lactose, sorbitol, or sucrose. The pH values of the spent media were measured at 16.5h, 40.5 h, and 64.5 h. The biofilm medium without carbohydrate, but with water instead served as the negative control. The biofilm groups without LGG (5SP) served as control groups.

Table 7. Strain composition of the biofilm groups, respective agars, and cultural conditions to detect viable cells from biofilms.

Group	Strain composition	Biofilm culture	Agars and culture
LGG	LGG	64.5 h, 37 °C, anaerobic condition, saliva-coated hydroxyapatite discs, media added and refreshed at 0, 16.5, and 40.5 h.	72 h, 37 °C, LGG: MRS in 5% CO_2, Ca: Sabouraud in air, Sm, Ss, Aa, Fn: BHI in anaerobic condition.
Ca	*C. albicans*		
Sm	*S. mutans*		
Ss	*S. sanguinis*		
Aa	*A. actinomycetemcomitans*		
Fn	*F. nucleatum*		
LGG+Ca	LGG, *C. albicans*		
LGG+Sm	LGG, *S. mutans*		
LGG+Ss	LGG, *S. sanguinis*		
4SP	*S. sanguinis*, *A. actinomycetemcomitans*, *C. albicans*, *F. nucleatum*		
4SP+LGG	*S. sanguinis*, *A. actinomycetemcomitans*, *C. albicans*, *F. nucleatum*, LGG		
5SP	*S. sanguinis*, *A. actinomycetemcomitans*, *C. albicans*, *F. nucleatum*, *S. mutans*		
5SP+LGG	*S. sanguinis*, *A. actinomycetemcomitans*, *C. albicans*, *F. nucleatum*, *S. mutans*, LGG		

4.2.3 Growth of pathogens in biofilms with probiotic LGG (II and III)

We cultivated the 16.5-h and 64.5-h biofilms of groups Sm+LGG, Ss+LGG, Ca+LGG, 4SP+LGG, and 5SP+LGG, and counted viable cell numbers of *S. mutans*, *S. sanguinis*, and *C. albicans* in each. The biofilms of groups Sm, Ss, Ca, 4SP, and 5SP served as control groups (**Table 7**).

The adhesion ratio and growth ratio of each strain we calculated by the equations in **Table 8**. The biofilms we stained with the LIVE/DEAD® kit or fluorescence *in situ* hybridization (FISH), and scanned with confocal laser scanning microscopy (CLSM) for biofilm structural analysis.

4.2.4 Growth of LGG in broth and biofilms (I-III)

Growth in broth with various carbohydrates (I)

The growth of probiotic LGG we investigated in both MRS broth medium and in biofilm medium with one sole carbohydrate, namely either fructose, glucose, lactose, sorbitol, or sucrose. Measurement of the optical density of each at 492 nm indicated growth status. The negative control was water.

Growth in biofilms with various species and with different carbohydrates

Growth of LGG in biofilms with various species compositions (II)

In 16.5-h and 64.5-h biofilms, we counted the viable cell numbers of LGG of groups Sm+LGG, Ss+LGG, Ca+LGG, 4SP+LGG, and 5SP+LGG. Group LGG served as the control. We also stained the biofilms with the LIVE/DEAD® kit and scanned them with CLSM for biofilm structural analysis.

Growth of LGG in biofilms with various carbohydrates (III)

In 64.5-h biofilms of group 5SP+LGG cultivated with one sole carbohydrate, namely either fructose, glucose, lactose, sorbitol, or sucrose, the viable cell number of LGG was counted for each. Water instead of carbohydrate served as the negative control. The adhesion ratio and growth ratio were calculated, and the biofilms were stained with FISH and scanned with CLSM for biofilm structural analysis.

4.2.5 Mouthwash effects on LGG-integrated biofilms (IV)

Figure 2 presents the three experimental designs of IV.

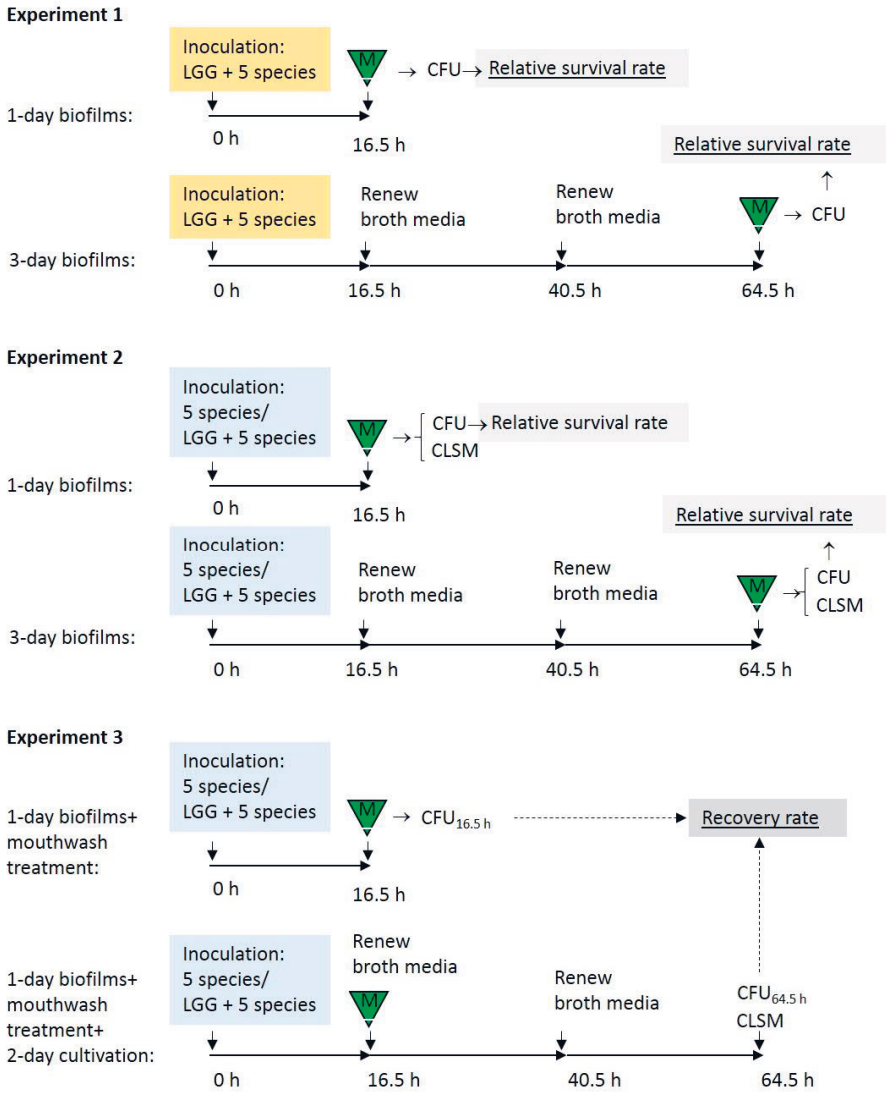

Figure 2. Design of Study IV. 5 species: a mixture of cell suspensions of *S. mutans*, *S. sanguinis*, *A. actinomycetemcomitans*, *F. nucleatum*, and *C. albicans*; LGG + 5 species: a mixture of cell suspensions of these five species and probiotic LGG (*L. rhamnosus* GG); triangle-M: one-minute mouthwash exposure; CFU: plate count to obtain colony-forming units; CLSM: biofilm structure analysis with confocal laser scanning microscopy. (To appear in the Open Access journal *Dentistry Journal*)

Pathogen responses to the mouthwash rinsing

Sensitivity of pathogens in LGG-integrated biofilms (Experiment 2)

The 16.5-h and 64.5-h biofilms of groups 5SP and 5SP+LGG underwent exposure to mouthwashes containing chlorhexidine gluconate, essential oils, and amine fluoride/stannous fluoride, each for one minute. Physiological saline served as the negative control. The viable cells of streptococci and *Candida* were counted and the relative survival rates calculated by the equation: relative survival rate = 100%×(CFU exposed to mouthwash)/(CFU exposed to saline). We then compared the relative survival rate of streptococci and *Candida* in biofilms with and without LGG (groups 5SP and 5SP+LGG). These biofilms we stained with the LIVE/DEAD® kit and scanned with CLSM for biofilm structural analysis.

Recovery of pathogens in LGG-integrated biofilms (Experiment 3)

The 16.5-h biofilms of groups 5SP and 5SP+LGG we treated with mouthwashes for one minute. We then counted the viable cells of streptococci and *Candida* immediately and after another 2-day cultivation. The recovery rates of streptococci and *Candida* during those 2 days we calculated by the equation: recovery $rate_{saline/Corsodyl®/Listerine®/Meridol®} = (\ln (CFU_{64.5}) - \ln (CFU_{16.5}))/48$ h, where $CFU_{64.5}$ was the viable cell number recorded after 2-day cultivation, $CFU_{16.5}$ was recorded immediately after the mouthwash treatment. The biofilms we stained with the LIVE/DEAD® kit and scanned with CLSM for biofilm structural analysis.

LGG responses to mouthwash rinsing (IV)

Sensitivity of LGG in the biofilms (Experiment 1)

The 16.5-h and 64.5-h biofilms of group 5SP+LGG were treated with mouthwashes. The viable cell numbers of LGG were plate counted and the relative survival rates calculated. The relative survival rate of LGG we then compared with that of streptococci and *Candida*. The biofilms we stained with the LIVE/DEAD® kit and scanned with CLSM for biofilm structural analysis.

Recovery of LGG in the biofilms (Experiment 3)

The 16.5-h biofilms of group 5SP+LGG we treated with mouthwashes. We counted the viable cell numbers of LGG immediately and then after another 2-day cultivation, with recovery rate calculated. The biofilms we stained with the LIVE/DEAD® kit and scanned with CLSM for biofilm structural analysis.

4.3 Experimental methods

4.3.1 Agar-overlay inhibitory assay (I)

The agar-overlay method originally described by Kékessy and Piguet [1970] was our choice to determine inhibitory activity. *Lactobacillus* cell suspension (2 µL) of lactobacilli we inoculated on a bottom-MRS agar containing one of the following carbohydrates: fructose, glucose, lactose, sorbitol, or sucrose. The pH of the bottom-MRS agar was adjusted to 5.5, 6.4, or 7.2. After cultivation of the lactobacilli anaerobically for 24 h at 37 °C, a soft Sabouraud agar with a cell suspension of *Candida* was poured over the bottom-MRS agar. After 24-h cultivation, the diameters of lactobacilli colonies and inhibition halo zones were measured and the effective inhibition ratio (equation appears in **Table 8**) calculated. Ratio scores below 0.5 are defined as slight inhibitory activity, between 0.5 and 1.5 as intermediate, and above 1.5 as strong.

4.3.2 Biofilm model (II-IV)

For the biofilm study, the model was adapted from Guggenheim *et al.* [2001] (the Zurich biofilm model) and Lemos *et al.* [2010]. In brief, each saliva-coated hydroxyapatite (HA) disc was placed in a vertical position in one well of a 24-well polystyrene cell culture plate with a single inoculation of pooled bacteria or yeast suspension or both at the very beginning. The microorganism actively adhered to the salivary pellicle and formed the biofilms. The biofilms we allowed to grow for 16.5 h or 64.5 h at 37 °C in anaerobic conditions. The biofilm medium, originally described by Lemos *et al.* [2010], was added at baseline (0 h). In biofilms grown for

64.5 h, the medium was refreshed at 16.5 h and 40.5 h by transferring the HA discs to new wells.

4.3.3 Plate counting (II-IV)

The viable cell number of microbial strains from biofilms were examined by plate counting. Biofilms on saliva-coated HA discs were washed down and sonicated into cell suspension. Serial dilutions of the suspension we cultivated on MRS, BHI, Brucella, or Sabouraud agars. For the agars and cultural conditions, see **Table 7**. Colony-forming units (CFU) of microbial strains were counted, and the numbers indicate their viable cell numbers in the biofilms.

In relation to the CFU, the adhesion ratio, growth ratio, relative survival rate, and recovery rate were calculated for each study. The equations are given in **Table 8**.

Table 8. Equations utilized in this project.

Ratio	Equation	Note	Study
Effective inhibition ratio (EIR)	EIR = (ID - CD)/CD	ID, diameter of inhibition halo; CD, diameter of *Lactobacillus* colony.	I
Adhesion ratio (AR)	AR = $CFU_{16.5}$/(ICC X ICV)	ICC, inoculated cell concentration; ICV, inoculated cell volume.	II
Growth ratio (GR)	GR = $CFU_{64.5}$/average of $CFU_{16.5}$	$CFU_{16.5}$, CFU acquired from 16.5-h bioflms; $CFU_{64.5}$, CFU acquired from 64.5-h biofilms.	II
Relative survival rate (RSR)	RSR = 100% X $CFU_{exposed\ to\ mouthwash}$ /$CFU_{exposed\ to\ saline}$	$CFU_{exposed\ to\ mouthwash/saline}$, CFU acquired from biofilms treated with mouthwash/saline.	IV

Ratio	Equation	Note	Study
Recovery rate (RR)	RR = (ln ($CFU_{64.5}$) - ln ($CFU_{16.5}$)) /(64.5 - 16.5)	$CFU_{16.5}$, CFU acquired from 16.5-h biofilms treated with mouthwashes; $CFU_{64.5}$, CFU acquired from 64.5-h biofilms treated with mouthwash at 16.5 h.	IV

4.3.4 Live/dead cell staining (II and IV)

Biofilms cultivated on saliva-coated HA discs were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (catalog number L7007, Molecular Probes™, Life Technologies™, Eugene, OR, USA) at room temperature. Syto 9 stained live cells, and propidium iodide dead cells.

4.3.5 Fluorescence *in situ* hybridization (III)

FISH served to analyze the biofilm structure. Biofilms were fixed with 4% paraformaldehyde, permeabilized, prehybridized in hybridization buffer, hybridized with fluorescently labeled oliogonucleotides (**Table 9**), washed, and stained with Hoechst 33342 (Thermo Scientific™, Rockford, IL, USA). The samples were embedded in Mowiol overnight at room temperature.

Table 9. Description and specificity of oligonucleotide probes in this study.

Probe	Label	Target bacteria	Target sequence (5´-3´)	Reference
Lcas467	Cy3	LGG	CCGTCACGCCGACAACAG	[Ardita *et al.*, 2014]
MUT590	Cy5	*S. mutans*	ACTCCAGACTTTCCTGAC	[Quevedo *et al.*, 2011]

4.3.6 Confocal laser scanning microscopy (II-IV)

By CLSM (Leica SP8, Leica Microsystems Gmbh, Wetzlar, Germany) we scanned the biofilm samples treated with LIVE/DEAD® kit and the FISH method. The images were obtained with a ×60 glycerol-immersion objective or a ×40 water-immersion objective. Each biofilm was scanned in randomly selected areas as a series of vertical optical sections; each section was 0.50 µm thick. The digital images were processed with software ImageJ or Fiji [Schneider *et al.*, 2012].

4.4 Statistical analyses

All experiments were carried out in triplicate, each experiment with two parallels. A significant difference was deemed as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Non-parametric Mann-Whitney U tests served for data in **Figures** 4-6 and **Tables** 11-12. One-way ANOVA and Duncan's tests were applied for data in **Table** 13.

5 Results

5.1 Inhibitory activity of probiotic lactobacilli against *Candida* (I)

The most pronounced inhibitory activity of the lactobacilli occurred in the presence of glucose and at pH 5.5. The EIRs of lactobacilli against *Candida* are in **Table 10**.

LGG, Shirota, SD2112, and CD2 were able to inhibit *C. albicans*. LGG produced a slight inhibitory effect on *C. glabrata*, but none of the lactobacilli had any effect on *C. krusei*. Because the highest EIR against *C. albicans* was detected with LGG. LGG and *C. albicans* were therefore selected for further testing in the following series of studies.

Table 10. The effective inhibition ratio of *Lactobacillus* to *Candida*, the bottom MRS agar with glucose, pH 5.5.

	C. albicans	*C. glabrata*	*C. krusei*
LGG	Intermediate[1]	Slight	N
Shirota	Intermediate	N	N
SD2112	Slight[2]	N	N
CD2	Slight	N	N
LB86	N[3]	N	N
LB LACT	N	N	N

[1] Intermediate: intermediate inhibitory activity, 0.5 < EIR < 1.5; [2] Slight: slight inhibitory activity, EIR < 0.5; [3] N: no inhibition.

5.2 Potential risk for dental hard tissues from lactobacilli (I-III)

5.2.1 Lactobacilli growing as planktonic cells (I)

A species-dependent change in pH was measurable. When lactobacilli were cultured in MRS broth containing one of the carbohydrates being tested, a species-dependent change in pH was detectable. The 24-h pH values of culture media are in **Figure 3**.

The lowest pH was for Shirota in fructose, glucose, and lactose, and for SD2112 in sucrose, and for LGG in sorbitol. Growth of LGG reduced the pH to 3.7-3.8 in fructose and glucose, to 4.4 in sorbitol, and to 5.4-5.5 in lactose and sucrose.

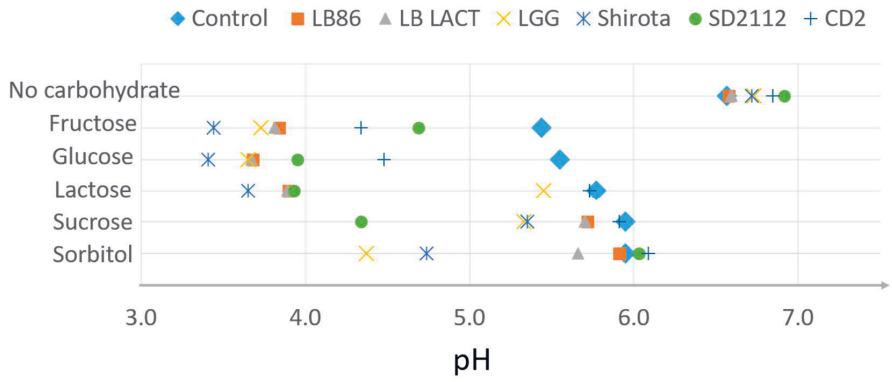

Figure 3. pH of spent media inoculated with *Lactobacillus* under various carbohydrate conditions.

5.2.2 Lactobacilli growing as biofilm cells (II and III)

Growth of LGG in biofilms with different species compositions (II)

The biofilm growth led to lowering the pH of the spent culture media at all three time points (**Figure 4**). The pH of groups LGG, Ca+LGG, and Ss+LGG was each all significantly ($p < 0.001$) lower than the pH of groups without LGG, namely Negative (the group inoculated with only saline), Ca, and Ss, at all three time points. The pH of group 5SP+LGG was significantly ($p < 0.05$) lower than that of group 5SP at 16.5 h. The pH of group 4SP+LGG was significantly ($p < 0.05$) lower than that of group 4SP at 64.5 h. No significant differences appeared between the pH of groups Sm and Sm+LGG ($p > 0.05$) at any time point.

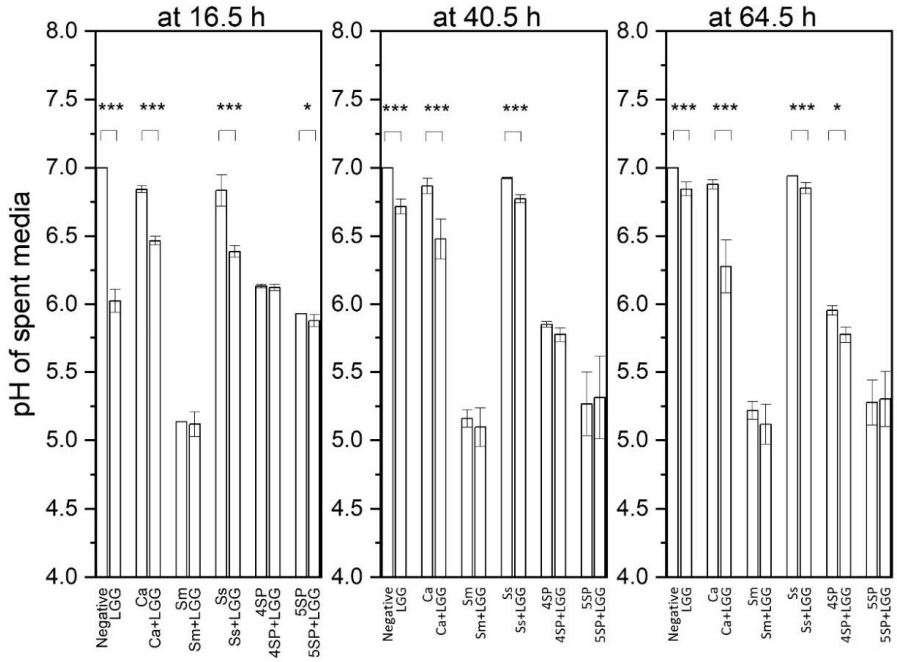

Figure 4. The pH of spent media in which biofilms grew. Negative: group inoculated with only saline, not with cell suspension.

Growth of LGG in biofilms with different carbohydrates (III)

No significant differences appeared between the pH of groups 5SP and 5SP+LGG at any time point with each of the carbohydrates ($p > 0.05$).

5.3 Growth of pathogens in biofilms with probiotic LGG (II and III)

The growth of pathogens in biofilms depended on species composition and was affected by the presence of LGG (**Table 11**).

The addition of LGG led to an increased adhesion ratio of *C. albicans*, but this difference fell short of significance ($p = 0.057$). The growth ratio of *C. albicans* was significantly reduced in group Ca+LGG (105.5 ± 40.9%) compared to that in group Ca (319.8 ± 133.4%) ($p < 0.05$).

The adhesion ratio of *S. mutans* was lower in group Sm+LGG (7.0 ± 2.2%) than in group Sm (24.8 ± 14.9%) ($p = 0.057$). A statistically significant difference ($p < 0.05$) in

the growth ratio of *S. mutans* was observable in group Sm+LGG (4490.1 ± 1338.6) compared to that in group Sm (918.3 ± 620.1), but no similar difference occurred in multi-species group 5SP+LGG compared to group 5SP ($p > 0.05$).

The adhesion ratio of *S. sanguinis* was higher in group Ss+LGG (1.6 ± 0.9) than in group Ss (0.1 ± 0.1) ($p < 0.05$), and in group 4SP+LGG this difference approached statistical significance ($p = 0.057$). The growth ratio of *S. sanguinis* was significantly higher ($p < 0.05$) in group Ss+LGG than in group Ss, but was significantly lower ($p < 0.05$) in the multispecies group without *S. mutans* (4SP+LGG) than in 4SP. No significant differences in adhesion ratio and growth ratio of *S. sanguinis* occurred in 5SP and 5SP+LGG ($p > 0.05$).

Table 11. Adhesion ratios and growth ratios of pathogens in biofilm groups cultivated with and without LGG.

Strain	Biofilm group	Adhesion ratio (%)	*p* value	Growth ratio (%)	*p* value
C. albicans	Ca	0.5 ± 0.2	0.057	319.8 ± 133.4	0.029*
	Ca+LGG	1.7 ± 1.4		105.5 ± 40.9 ↓	
	4SP	0.3 ± 0.3	0.200	710.5 ± 328.5	0.486
	4SP+LGG	1.0 ± 0.7		463.5 ± 283.0	
	5SP	0.6 ± 0.3	0.686	1612.5 ± 715.6	0.486
	5SP+LGG	1.5 ± 2.0		1172.8 ± 696.9	
S. mutans	Sm	24.8 ± 14.9	0.057	918.3 ± 620.1	0.029*
	Sm+LGG	7.0 ± 2.2		4490.1 ± 1338.6 ↑	
	5SP	20.8 ± 9.6	1.000	12006.7 ± 6645.7	0.886
	5SP+LGG	27.5 ± 21.6		10578.2 ± 5402.7	
S. sanguinis	Ss	0.1 ± 0.1	0.029*	15.3 ± 9.2	0.029*
	Ss+LGG	1.6 ± 0.9 ↑		58.7 ± 27.4 ↑	
	4SP	22.7 ± 7.7	0.057	1210.3 ± 498.7	0.029*
	4SP+LGG	42.8 ± 7.7		189.8 ± 129.7 ↓	
	5SP	37.2 ± 28.1	1.000	358.1 ± 415.6	0.343
	5SP+LGG	35.1 ± 21.7		377.6 ± 192.4	

Cell adhesion ratio of each strain to saliva-coated HA discs at their adhesion stage; Viable cells' growth ratio for each strain in each group at their self-development stage; ↑/↓: higher/lower than LGG-free group; Data represent means ± SDs. *$p < 0.05$.

5.4 Growth of probiotic LGG in broth and biofilms (I-III)

5.4.1 LGG grown in broth medium (I and III)

The carbohydrate utilization profile of LGG in biofilm medium was similar to that in MRS broth. LGG grew with glucose, fructose, and sorbitol but not with lactose or sucrose. The maximum optical densities measured for biofilm culture media and MRS broth in the presence of LGG were 0.1455 (at 490 nm) and 1.137 (at 492 nm).

5.4.2 LGG grown in biofilms

LGG grown with different strains (II)

LGG showed better growth in all dual- and multi-species biofilm groups than in group LGG ($p < 0.05$), except in group Ss+LGG which fell short of significance ($p = 0.057$) (**Table 12**). The adhesion ratio of LGG in group 4SP+LGG was significantly higher than that in group LGG ($p < 0.05$).

Table 12. The adhesion ratio and growth ratio of LGG cultivated with different species.

Biofilm group	Adhesion ratio of LGG	*p* value	Growth ratio of LGG	*p* value
LGG	10.5 ± 6.2	-	6.7 ± 3.8	-
Ca+LGG	12.0 ± 5.5	1.000	944.6 ± 579.5 ↑	0.029*
Sm+LGG	13.2 ± 2.1	0.343	32.3 ± 17.6 ↑	0.029*
Ss+LGG	11.8 ± 9.7	1.000	16.5 ± 7.7	0.057
4SP+LGG	23.4 ± 4.4 ↑	0.029*	566.8 ± 217.2 ↑	0.029*
5SP+LGG	7.0 ± 3.3	0.686	215.3 ± 108.5 ↑	0.029*

Cell adhesion ratio of LGG to saliva-coated HA discs at adhesion stage; Viable cells growth ratio of each strain in each group at their self-development stage; ↑/↓: higher/lower than Group LGG; Data represent means ± SDs, *$p < 0.05$.

Growth of LGG with various carbohydrates in 5SP+LGG (III)

The growth of LGG in biofilm group 5SP+LGG varied, depending upon the presence of various carbohydrates (**Table 13**). In these multi-species biofilms, LGG grew

significantly better in the presence of fructose, glucose, lactose, and sucrose when compared to the presence of sorbitol ($p < 0.05$). When no carbohydrate was added to the medium, LGG was still able to produce biofilms.

Table 13. Viable cell number of LGG from 64.5-h biofilms group 5SP+LGG cultivated with a sole carbohydrate.

Carbohydrate group	Viable cells of LGG (Log_{10} CFU/disc)
Negative	3.474 ± 0.337 [a]
Sorbitol	5.166 ± 0.178 [b]
Fructose	5.966 ± 0.262 [c]
Glucose	6.296 ± 0.308 [c]
Lactose	5.860 ± 0.206 [c]
Sucrose	6.335 ± 0.174 [c]

Negative: carbohydrate free; Different small letters represent a significant difference, $p < 0.05$.

5.5 Mouthwash effects on pathogens in biofilms with probiotic LGG (IV)

5.5.1 Relative survival rates of streptococci and *Candida*

The relative survival rates of *S. mutans*, *S. sanguinis*, and *C. albicans* in 1- and 3-day biofilm group 5SP+LGG did not significantly differ from that in group 5SP (**Figure 5**).

Figure 5. Relative survival rates of *S. mutans*, *S. sanguinis*, and *C. albicans* in 1- (left) and 3-day (right) LGG-free (-LGG) and LGG-integrated (+LGG) biofilms. Values as diamonds. The dotted line is the relative survival rate that equals 100%. No significant difference observable ($p > 0.05$). (To appear in the Open Access journal *Dentistry Journal*)

5.5.2 Recovery rate of streptococci and *Candida*

The recovery rates of *S. mutans*, *S. sanguinis*, and *C. albicans* in group 5SP+LGG after mouthwash rinsing did not differ significantly from those in group 5SP (**Figure 6**).

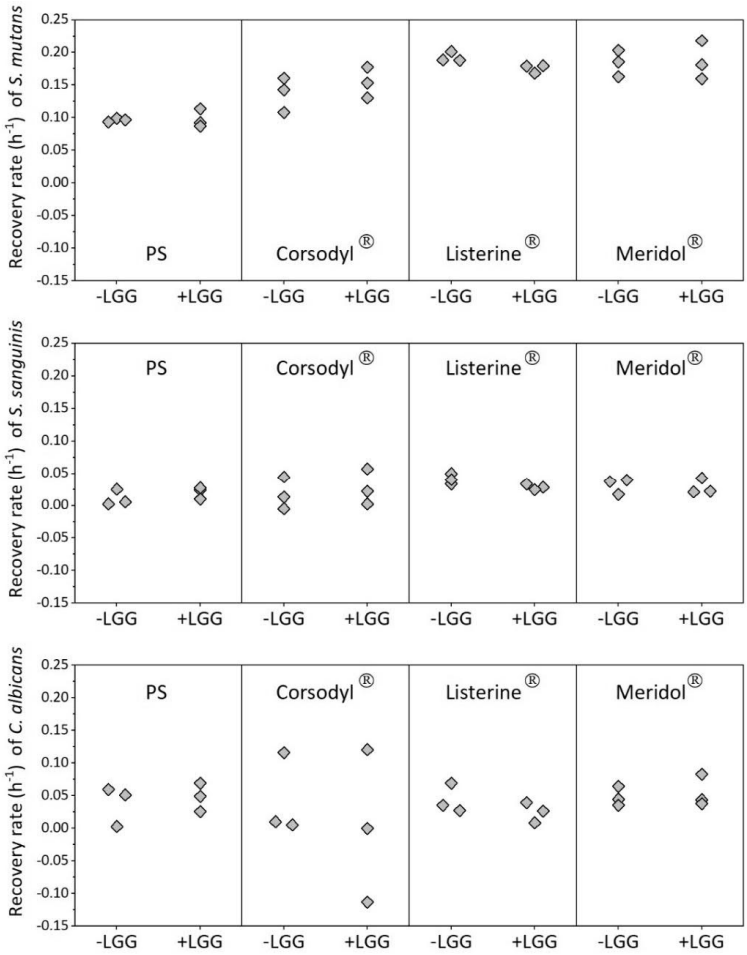

Figure 6. Recovery rates of *S. mutans*, *S. sanguinis*, and *C. albicans* in LGG-free (-LGG) and LGG-integrated (+LGG) biofilms after mouthwash rinsing. Values as diamonds. PS, physiological saline. No significant difference observable ($p > 0.05$). (To appear in the Open Access journal *Dentistry Journal*)

6 Discussion

6.1 Inhibitory activity of probiotic lactobacilli against *Candida* (I)

To evaluate the inhibitory activity of lactobacilli against *C. albicans* and non-*albicans Candida*, the agar-overlay assay was employed with various pH and single carbohydrate in the agar layer at the bottom. Four species of the lactobacilli showed inhibitory effects: effects occurring against *C. albicans*; a slight LGG inhibition of *C. glabrata*; but no lactobacilli species inhibition of *C. krusei*. Under the conditions of this study, the *Lactobacillus* strains showed a stronger inhibitory effect against *C. albicans* than against non-*albicans Candida*.

Jorgensen *et al.* [2017] provided similar findings that *L. reuteri* showed better inhibitory activities against *C. albicans* than against *C. krusei*, and this applied both to clinical isolates and to reference *Candida* strains. The non-*albicans Candida* appeared in its host after administration of antifungal drugs, which makes it unsurprising to find stronger survivability of non-*albicans Candida*. Nevertheless, it may appear that non-*albicans Candida* is not susceptible to lactobacilli. Bulgasem *et al.* [2016] isolated new potential lactic acid bacteria from honey that shows an even stronger inhibition activity against *C. krusei* than against *C. albicans*. Furthermore, in a spot overlay assay, in a plate-based microtiter assay, and in one biofilm model, vulvovaginal-candidiasis-causing *C. glabrata* has been inhibited by *L. rhamnosus* GR-1 and *L. reuteri* RC-14 [Chew *et al.*, 2015a, b]. The reasons why the inhibition of probiotic lactobacilli showed different effects on different species of *Candida* are still unknown.

Data from some clinical trials have demonstrated certain inhibitory activities of probiotic lactobacilli, *Bifidobacterium*, *Propionibacterium,* and *Streptococcus* against *Candida*, but the intake of probiotic *L. reuteri* or *S. salivarius* has shown no effect on the species profile of *Candida* in caries-active children or in participants with systemic oral lichen planus [Burton *et al.*, 2013; Keller and Kragelund, 2018]. Mendonca *et al.* [2012] observed a minor reduction in numbers of non-*albicans*

species in the mouths of elderly individuals after their consumption of a probiotic mixture of *L. casei* and *B. breve*.

6.2 Potential risk of lactobacilli for dental hard tissues (I-III)

The second aim was to evaluate the potential risk that lactobacilli may impose on dental hard tissue by their lowering the oral environment's pH. We added various sugars to the culture medium and then compared the pH of spent culture media when lactobacilli were grown in MRS broth or in a single- or multi-species biofilm model.

The acid-producing ability of lactobacilli was confirmed, indicating that the pH alterations by the addition of LGG were more pronounced in mono- and dual-species biofilm groups, but neither in multi-species biofilms nor in those cultured with *S. mutans*. Madhwani and McBain [2011] reported similar results, finding that the exposure of *L. reuteri* on salivary biofilm microcosms in an HA disc model and mature continuous culture plaques with constant-depth film fermenters caused no significant pH decreases. Pham *et al.* [2011] also reported that LGG did not affect the pH when cultured with *S. mutans* in dual-species biofilms or in saliva-derived microcosms. In contrast, another *in vitro* dental biofilm model provided support for the finding that LGG co-cultured with *S. mutans* for 10 days led to significant mineral losses in dental tissue in comparison to results for tissue exposed to biofilms containing *S. mutans* or LGG alone [Schwendicke *et al.*, 2014].

This discrepancy between study results may be attributable to differences in culture media or in the biofilm species. Schwendicke *et al.* [2014] cultivated their biofilms with BHI containing 2% sucrose, a nutrient-rich medium. This medium enabled both LGG and *S. mutans* to grow well with less competition; along with their growth, acids in the broth and the mineral loss increased. Some studies [Madhwani and McBain, 2011; Pham *et al.*, 2011], however, utilizing artificial saliva medium, which is similar to the nutritional environment of human saliva, showed no pH decrease in the culture, which is consistent with the findings of this thesis. In such nutrition-depleted media, the biofilm strains may need to compete with the surrounding

species for nutrients, and the final pH depends on the energy sources available. Second, the biofilm species also influenced the final pH. In an environment with limited nutrition, the microorganisms cultured with other species were more liable to make full use of nutrients. They proliferated better, and they created more types of acids or metabolites than were evident in mono- and dual-species groups. Consequently, the acids or metabolites could be digested by the synergistic effect in a microbial environment [Schink, 2002].

To conclude, in this *in vitro* study, the addition of LGG did not lead to increased potential risk for dental hard tissues in a multi-species group with the growth media that we tested.

6.3 Growth of pathogens in biofilms with probiotic LGG (II and III)

In our investigation of the inhibition by LGG of oral pathogenic strains in dual- and multi-species biofilm groups, LGG reduced the growth of *C. albicans* in all biofilm groups, reduced the adhesion ratio of *S. mutans* in the dual-species biofilm group, and reduced the growth ratio of *S. sanguinis* in the biofilm group 4SP+LGG.

Our results agree with previous *in vitro* reports that *Lactobacillus* strains inhibit the biofilm development of *C. albicans* [Song and Lee, 2017; Rossoni *et al.*, 2018a]. Moreover, the short-term administration of single and multi-species probiotic lactobacilli have lowered *Candida* counts in the elderly and in individuals treated with *Candida*-associated stomatitis [Hatakka *et al.*, 2007; Li *et al.*, 2014; Ishikawa *et al.*, 2015; Kraft-Bodi *et al.*, 2015]. The underlying mechanisms of inhibitory activity are not yet fully understood, however. Basson [2000] noted that in a chemostat with mixed microbial species under both glucose-limited and -excess conditions, *L. casei* consumed glucose faster than did *C. albicans*. Recent studies [James *et al.*, 2016; Ribeiro *et al.*, 2017; Rossoni *et al.*, 2018a; Tan *et al.*, 2018] reported that the supernatant of lactobacilli showed similar effects as did lactobacilli cells in reducing the biofilm formation of *C. albicans* and non-*albicans Candida*, and it downregulated the expression of *C. albicans* genes (ALS3, HWP1, EFG1, and CPH1) involved in the biofilm formation.

These findings confirmed the beneficial effects of the extracellular metabolites of lactobacilli. Probiotic lactobacilli have also shown the ability to reduce the virulence of *Candida*, including reduction in adhesion, invasion, and hyphal extension [Matsubara *et al.*, 2016; Mailander-Sanchez *et al.*, 2017]. Mailander-Sanchez *et al.* [2017] also further demonstrated, first, that LGG did not directly affect the viability of *C. albicans*; second, glucose depletion dramatically altered the composition of the cell membranes and caused reorganization of organelles; third, the synergy effects of glucose depletion and reduced adhesion induced a reduction in *C. albicans*-caused epithelial damage. However, in our study, LGG caused a slight increase in the adhesion ratio of *C. albicans* in all biofilm groups, possibly because of the cell concentration of LGG, which was not high enough at the adhesion stage to produce a functional concentration of extracellular metabolites able to hinder the adhesion of *C. albicans*. Another mechanism may be via some extracellular metabolite utilized by *C. albicans* that improved the *C. albicans* adhesion ratio.

A reduction in the *S. mutans* adhesion ratio in the present experimental group Sm+LGG emerged to a greater extent than in the group Sm. Other studies [Chung *et al.*, 2004; Tahmourespour *et al.*, 2011; Lee and Kim, 2014; Savabi *et al.*, 2014; Wu *et al.*, 2015; Ciandrini *et al.*, 2016; Wasfi *et al.*, 2018] have shown that the addition of probiotic *Lactobacillus*, its cell-free supernatant, and its biosurfactants downregulates the expression of genes encoding glucosyltransferases (*gtf*B and *gtf*C) and fructosyltransferase (*ftf*). These are associated with biofilm formation by *S. mutans*, and therefore inhibit the adhesion of *S. mutans*.

Interestingly, the growth ratio of *S. mutans* was more significantly enhanced in our experimental group Sm+LGG than in the group Sm. This result is consistent with findings [Wen *et al.*, 2010] that biofilm formation by *S. mutans* was modestly increased when it was co-cultured with *L. casei* in dual-species biofilms. This result may be related to the metabolites of lactobacilli, which can be consumed also by *S. mutans*. However, in our study, the reduced adhesion ability and increased growth of *S. mutans* coinciding with the addition of LGG to the dual-species group did not appear in the multi-species group. The reason may be that in the multi-species

ecosystem, various bacterial and yeast cells were interacting with each other, the effect of LGG was diluted by effects of the other microorganisms, or the other stains each had a stronger influence than that of LGG. Similar results also appear with *S. sanguinis*, whose growth LGG inhibited in the experimental group 4SP+LGG compared with its growth in group 4SP, but these effects disappeared in group 5SP+LGG when compared with the effects in group 5SP. *S. sangunis* may thus be influenced more by *S. mutans* than by LGG. The underlying mechanisms still need further exploration, however.

To conclude, LGG inhibited biofilm formation by *C. albicans* in all experimental settings, but the effects of LGG on *S. mutans* and on *S. sanguinis* varied depending on the group composition.

6.4 Growth of probiotic LGG in broth and biofilms (I-III)

Our results showed that LGG growth was promoted in all the dual- and multi-species groups tested and that the highest growth ratio occurred in the experimental group Ca+LGG.

LGG growing alone in the culture did not show good biofilm-forming ability, but its growth was greater in the presence of *S. mutans*, *S. sanguinis*, *C. albicans* and in the multi-species community. These findings agree with previous ones [Filoche *et al.*, 2004; Pham *et al.*, 2009; Wen *et al.*, 2010; Wen *et al.*, 2017]. In dual-species biofilms, *S. mutans* has led to increased biofilm growth of *L. casei*, *L. rhamnosus*, *L. plantarum*, and also *L. gasseri*, but not the growth of *L. fermentum* [Filoche *et al.*, 2004; Wen *et al.*, 2010; Wen *et al.*, 2017]. *L. salivarius* W24 has also shown better biofilm growth in a saliva-derived microbial community *in vitro* [Pham *et al.*, 2009]. One possible reason may be that *S. mutans* and other microorganisms adhered to the salivary pellicle and provided more binding sites to the later colonizer, LGG. The other possibility may have been secondary metabolites generated by *S. mutans* and other microorganisms that could stimulate the growth of LGG.

Our study further indicates that the viable cell population of LGG in biofilm formation was substantially enhanced in the presence of *C. albicans* cells alone or

with its cell-free culture supernatant, or with them both. This result is in contrast to an observation by Liu and Tsao [2009], that yeast addition did not affect the growth of *L. rhamnosus* DR20 in fermented milk. They also showed that *C. kefyr* NCYC 143, *C. krusei* MUY-14, and other yeasts improved the survival of *L. rhamnosus* DR20 in fermented milk and yoghurt. Such a discrepancy may again be attributable to the culture media. The latter study used whole milk as a medium, whereas for our study we chose a nutrient-depleted medium. The metabolites of *Candida* may therefore also benefit the growth of *Lactobacillu*s. Nevertheless, numerous studies and patents have provided evidence of the enhanced stabilization of lactobacilli with the aid of yeast. For instance, the addition of dried non-viable yeasts has served to maintain the stability of dried viable lactic acid bacteria such as *L. acidophilus* [Hsia, 2001].

In summary, the present results show enhanced biofilm growth of LGG when streptococci, *Candida*, and multispecies were present.

6.5 Mouthwash effects on LGG-integrated biofilms (IV)

Evaluation of mouthwashes in regard to the sensitivity to and recovery of microorganisms in the multi-species biofilms showed that all the mouthwashes clearly inhibited LGG. Its recovery was slower than that of the streptococci and *Candida*. LGG showed no influence upon the response of the streptococci and *Candida* to the mouthwash rinsing, however.

When treated with the mouthwashes, LGG in the biofilms died easily and recovered slowly. Earlier studies have also indicated that *Lactobacillus* species is sensitive to mouthwashes [Malhotra *et al.*, 2011; Yousefimanesh *et al.*, 2015; Oliveira *et al.*, 2017]. Some researchers have, however, also shown the opposite *in vitro* results [McDermid *et al.*, 1987; Zheng and Wang, 2011; Evans *et al.*, 2015] and *in vivo* results [Sari and Birinci, 2007; Aminabadi *et al.*, 2011]. The reasons for these differences are unknown, but indicate that mouthwashes and the administration of probiotics should not take place simultaneously. The active ingredients in mouthwashes indiscriminately killed the microorganisms, and the recovery of the

surviving LGG was too slow to show any further beneficial effects. Hence, a practical suggestion for the consumer may be to take probiotics only following any mouthwash rinse.

7 KEY FINDINGS AND CONCLUSIONS

The present results imply that probiotics can suppress the growth of certain pathogens under proper conditions, that they should not affect dental health detrimentally, and that they do not interfere with the use of mouthwashes. The main findings can be summarized as follows:

1. The capabilities of probiotic lactobacilli in inhibiting the growth of *C. albicans* were strain-dependent and modified by pH and sugars. No inhibition against *C. glabrata* and *C. krusei* occurred.
2. The probiotic LGG did not significantly reduce the pH when cultivated with the other five species of oral pathogens in multi-species biofilm models. None of the sugars tested changed these results.
3. The probiotic LGG led to increased growth of *S. mutans* and *S. sanguinis* compared with their growth without LGG, but inhibited the growth of *C. albicans*. LGG had no impact on the growth of the pathogens in the multi-species biofilms, but it reduced the growth ratio of *S. sanguinis* in the absence of *S. mutans.*
4. Probiotic LGG in multi-species biofilms was able to survive and grew well with lactose or sucrose as its only carbohydrate source.
5. LGG neither enhanced nor weakened the antimicrobial effects of mouthwashes on the pathogens. The residual LGG had no effect on pathogens' recovery.
6. *C. albicans* significantly elevated the growth level of LGG in biofilms.

Based on these results, it will be interesting to further study whether the colonization of probiotic lactobacilli remains longer in individuals with *C. albicans* than in those lacking *C. albicans*, which finding may benefit patients with oral yeast infections. In addition, it is essential to find a proper delivery system of probiotic administration in the oral cavity to ensure long-lasting effects.

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